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SURPLUS PHOSPHORUS UPTAKE BY MICROORGANISMS. BATCH TESTS WITH DILUTE ACTIVATED SLUDGE CULTURES

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Texas University
Austin, Texas

February 1969

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Surplus Phosphorus Uptake By Microorganisms

Batch Tests with Dilute Activated Sludge Cultures

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SURPLUS PHOSPHORUS UPTAKE BY MICROORGANISMS--
Batch Tests with Dilute Activated Sludge Cultures

Technical Report No. 2
to the
Federal Water Pollution Control Administration
Department of Interior
Grant WP-01449-01

by

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ENVIRONMENTAL HEALTH ENGINEERING RESEARCH LABORATORY

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February, 1969

ABSTRACT

An alkaline phosphatase assay based on the use of a p-nitrophenyl phosphate substrate was studied for its applicability to indicate "surplus" phosphorus uptake by a dilute heterogeneous bacterial culture obtained from an activated sludge process. This investigation demonstrated the validity of the enzymatic bioassay using viable bacterial cells and determined conditions under which the bioassay should be conducted as a standard procedure. Alkaline phosphatase activity was found to be repressed by both orthophosphate external to the cells and by stored phosphorus within the bacterial cells. Based on these studies, the assay appears useful to help elucidate the mechanism responsible for the abnormally high phosphorus removal being attained by various activated sludge plants in the United States. The research also indicates the possible significance of the surplus phosphorus uptake phenomenon in waste treatment processes.

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Introduction to the Problem

The water quality of a number of natural and man-made impoundments is currently deteriorating rapidly because of increased algal and plant growth. An increase in the phosphorus content of these waters frequently has been cited as the cause for such changes. One means of controlling the phosphorus concentration in impoundments is to remove this nutrient from wastewater effluents. Many physical and chemical tertiary treatment methods for phosphorus removal are too costly at the present time for ordinary socio-economic circumstances. Thus, considerable study has been undertaken in recent years on biological methods of phosphate removal.

Borchardt and Azad (1967) indicated that algae, under certain circumstances, were capable of removing phosphate from solution in quantities greater than that required for growth. The quantity of removed phosphorus which is greater than that needed for growth is called "surplus" phosphorus. Using algae to remove phosphorus, however, is difficult because of unfavorable climatic conditions for algal growth in many parts of the world and because of the present lack of feasible methods for harvesting the algae.

Considerable work has also been conducted on the use of the activated sludge processes to remove phosphorus. In normal sewage treatment practice, little phosphorus is usually removed because of the low carbon to phosphorus ratio (Wuhrman, 1957). Srinath, et al (1959) first reported that during aeration of mixed liquor an abnormally rapid uptake of soluble phosphorus occurred. He pointed out the biological nature of this phosphate uptake by demonstrating the marked decrease in phosphate removal upon addition of mercuric chloride and upon heating the sludge at 40°C. Later, Feng (1962) also reported on the ability of activated sludge to remove concentrations of orthophosphate much higher than those generally

accomplished in practice. Phosphorus accumulation in activated sludge was indicated by Horn, et al (19[illegible]) who monitored a treatment process and found that the concentrated activated sludge had an average total phosphate content of 7 per cent on a dry weight basis (as P).

Levin and Shapiro (1965) obtained similar results and showed that the removal was biological because of decreased phosphate removal upon addition of 2, 4-dinitrophenol which inhibits oxidative but not substrate phosphorylation. These investigators concluded from their tests that "surplus" phosphorus uptake had occurred Duguid, et al (1954), Smith, et al (1954) and Sall, et al (1956) have demonstrated that phosphorus can accumulate in the form of metachromatic granules (volutin). Levin and Shapiro (1965) also stated that "surplus" phosphorus uptake was a function of oxygen concentration. Hall and Engelbrecht (1967) reported phosphorus removal dependent on oxygen concentration and found DO levels of approximately 2 mg/l necessary for optimum soluble phosphorus uptake. Ryckman and Rains (1968) pointed out, however, that all Levin and Shapiro (1965) demonstrated was an increased orthophosphate removal with increased aeration rate. Ryckman and Rains (1968) stated that mixing was more important. Yet, Vacker, et al (1966) reported that the main difference in phosphorus removal for three San Antonio plants was the fact that the plant with the 96 per cent phosphorus removal rate had a significantly higher dissolved oxygen concentration at the end of the aeration tank in comparison to the other two plants. Scalf, et al (1968) monitored an activated sludge plant and found phosphate removal in the aeration tank of greater than 90 per cent. High dissolved oxygen levels in the aeration tank effluent and, hence, in the final clarifiers (3 mg/l) seemed to prevent desorption of phosphate from sludge solids to final effluent. Horn, et al (1968) also reported highly efficient phosphorus removal in aeration tanks, but a lowering of overall efficiency due to desorption in the final clarifiers. This was attributed to excessive detention

time and insufficient dissolved oxygen concentrations in the clarifiers. Scalf, et al (1968) indicated, further, in pilot studies, that O_2 demanding substrate additions could increase initial desorption of phosphate in mixed liquor. However, Menar and Jenkins (1968) reported that this release of phosphorus from the activated sludge was due to a dissolution of a calcium phosphate precipitate caused by a decrease in pH at the entrance to the aeration tank.

Srinath, et al (1967) showed that the efficiency of the bacteria isolated from an activated sludge in the removal of ^{32}P from sewage was poor. They reported that the rapid removal of ^{32}P was largely due to the Vorticellid protozoa, such as Epistylis sp. in the activated sludge. The factors which adversely affected the activated sludge in the removal of ^{32}P also affected the protozoan. After removal, a small part of the ^{32}P dialysed out over a period of 24 hours.

Vacker, et al (1966) also noted that the hardness of the San Antonio effluent was lower than that of the raw wastewater. However, they concluded that cationic precipitation could not account for the major part of the phosphorus reduction. Levin and Shapiro (1965) reported in their studies that above pH 9.0 the quantity of phosphorus removed decreased and, thus, also concluded that the removal could not be chemical. It should be noted, however, that in the pH studies of Levin and Shapiro (1965) the activated sludge had not been previously acclimated to the various pH conditions. Furthermore, Menar and Jenkins (1968) have hypothesized the possibility of a calcium phosphate precipitation at a much lower pH than 9.0. They also postulated that the increased aeration rate effect on phosphorus removal reported by Levin and Shapiro (1965), as well as by Vacker, et al (1966), was due to a decrease in CO_2 concentration at the tail end of the aeration unit caused by air stripping and therefore, an increase in the pH of the mixed liquor. Furthermore, Jenkins and Menar (1967) reported removals of phosphorus

in activated sludge units that did not vary significantly from 20 per cent of the influent phosphorus concentration until substrate removal rates were decreased below 0.3 lb. COD removed/lb. VSS/day, even though conditions that have been reported necessary for "surplus" phosphorus uptake were maintained.

Priesing (1967) reported on studies at a number of wastewater treatment plants in which changes of a number of operating conditions greatly affected phosphorus removal. He concluded that the mechanism of removal involved adsorption of orthophosphate under aerobic conditions, but made no conclusion concerning later metabolic utilization of the adsorbed orthophosphate by the cells. Ryckman and Rains (1968) also indicated a physical sorption mechanism. Hall and Engelbrecht (1967), however, reported no effect of mixed liquor suspended solids concentration within a range of 750 to 8000 mg/l on the rate or extent of phosphorus removal. Myers, et al (1968), on the other hand, demonstrated an optimum total suspended solids concentration for orthophosphate removal in the range of 2600 - 5000 mg/l. According to Scalf, et al (1968), a mixed liquor suspended solids concentration between 1200-6000 mg/l resulted in efficient PO_4 removal by activated sludge in pilot studies.

After reviewing the literature, it is obvious that the activated sludge process under some operating conditions can remove large quantities of phosphorus not usually attained in normal sewage treatment processes. However, there is basic disagreement on the mechanism of removal. All that is usually measured is the concentration of the phosphorus remaining in the solution, the quantity of phosphorus in the activated sludge, and variables such as aeration rate, oxygen concentration, biological solids, pH, etc. that are believed to be related to phosphorus removal. In order to distinguish between different mechanisms of removal so as to regulate the actual activated sludge process to obtain optimum phosphorus removal a technique is needed which indicates what is occurring within the bacterial cell.

An Enzymatic Method to Detect "Surplus" Phosphorus Uptake

The following enzymatic reaction depicts the liberation of p-nitrophenol from an ester, p-nitrophenyl phosphate (PNP), by non-specific alkaline phosphatase:

$$\underset{\text{(colorless)}}{\text{p-nitrophenyl phosphate}} + H_2O \xrightarrow[35^{\circ}C]{\text{alkaline phosphatase}} \underset{\text{(yellow)}}{\text{p-nitrophenol}} + H_3PO_4$$

Alkaline phosphatase is produced in abundance when inorganic phosphorus in the solution is limiting to the growth of microorganisms. If inorganic phosphorus is present, the enzyme is repressed (Stadtman, 1961).

Fitzgerald and Nelson (1966) suggested that phosphorus stored by algae in excess of metabolic needs would also repress the production of alkaline phosphatase when the algae were placed in a medium containing PNP with no other source of phosphate. They assumed that if there were no "surplus" phosphorus storage by the algae, than an abundance of alkaline phosphatase would be produced upon transfer to the PNP substrate. These investigators used the orthophosphate extracted by a hot water leaching of the algal cells as a measure of phosphorus storage. Algal cultures that were grown in solutions containing high phosphorus concentrations were found to have high orthophosphate extractions and low enzymatic activity when transferred to the PNP substrate. Algae cultured in solutions with low phosphorus concentrations had low orthophosphate extractions and a hgih enzymatic activity. Fruh and Pessoney (1969) evaluated the above procedure of Fitzgerald and Nelson (1966) and found it exceptionally useful for axenic algal cultures and for algal samples from various Texas aquatic environments. They also reported that a total inorganic phosphate extraction was more reliable

than the orthophosphate extraction proposed by Fitzgerald and Nelson (1966) as an indication of phosphorus storage. Moore and Fruh (1969) reported that the mechanism of enzyme repression was the same whether the phosphorus compound was orthophosphate, metaphosphate or organic phosphorus. Furthermore, these investigators found that the PNP concentration used had to be significantly higher than that reported by previous investigators so that maximum enzymatic activity could be attained. All of these investigators used suspended solids as a measure of living algal mass.

Torriani (1960) showed the influence of inorganic phosphate on the formation of phosphatases, both alkaline and acid, by Escherichia coli. The two bacterial phosphatases differed markedly in their substrate specificity and their condition of formation. Whereas acid phosphatase was always present in small quantities, alkaline phosphatase was only present when inorganic phosphorus was limiting growth. The alkaline phosphatase activity was demonstrated to have maximum activity between pH 8.8 and 9.8. At pH values below 7.0, the alklaine phosphatase activity decreased markedly and approached zero activity at pH 5.3. When inorganic phosphorus was present in excess of metabolic needs and E. coli growth occurred at pH 7.0 or above, no alkaline phosphatase activity was found and no hydrolysis of phosphomonesters (such as p-nitrophenyl phosphate) present occurred. The inorganic phosphorus was used preferentially until its concentration became limiting. At that time, alkaline phosphatase activity started and increased with time.

The objective of this study was to determine if the alkaline phosphatase activity measurement can be used as an indicator of "surplus" phosphorus uptake by a mixed culture of bacteria obtained originally from an activated sludge unit.

Experimental Procedure

The bacterial cultures used in this study were obtained from the activated sludge unit at the City of Austin's Govalle Sewage Treatment Plant. This "main unit" culture was fed once a day with a substrate whose components were similar to the substrate used in later experiments. During an experiment a plexiglass "batch unit" which consisted of five compartments was inoculated with bacteria from the "main unit." Only a small quantity of bacteria from the "main unit" was used to prevent a carry-over of phosphorus. All units were aerated at a rate sufficient to maintain completely mixed conditions as well as saturating oxygen concentrations. No precipitation occurred in any units when bacteria were not present.

At first, suspended solids measurement was used to indicate bacterial growth. Eight replicates of a suspended solids measurement averaged 279 mg/l with a standard deviation of 13 mg/l. A correlation was obtained between optical density at 400 mμ and suspended solids. This correlation was used throughout all experiments as a check on suspended solids determinations. Later, volatile suspended solids were used when the suspended solids measurement was found to be an unreliable measurement of bacterial growth in other than the exponential growth phase.

A sample of the culture upon which a measurement of the alkaline phosphatase activity was desired was withdrawn from a batch unit and filtered through a prewashed 0.45 μ membrane filter. One washing with 10 milliliters of distilled water was required to remove the phosphorus occluded within the bacterial mass on the filter. After washing, the bacteria were placed in a beaker containing 100 milliliters of the p-nitrophenyl phosphate (PNP) substrate which was kept well mixed and incubated at 35°C. The components of the PNP substrate were exactly

the same as the batch substrate from which the culture was obtained except that there was no phosphorus source other than the PNP. From a calibration curve using a wavelength of 410 mμ, the concentration of p-nitrophenol liberated at various incubation times was measured. (Sigma Technical Bulletin, 1963). At the same time at which a portion of the sample was being taken from the batch unit for the enzyme test, suspended solids or volatile suspended solids measurements were made on a second portion of the sample. The enzymatic activity was then recorded as μmoles p-nitrophenol liberated per liter/mg solids/hr. Based on such units, comparison could be made to the alkaline phosphatase activity found for algae by Fitzgerald and Nelson (1966) and Fruh and Pessoney (1969).

The total soluble phosphorus remaining in the filtrate was measured using a modification of the Lundgren (1960) autoanalyzer method and a modification of the persulfate oxidation method (Menzel and Corwin, 1965; Gales, et al, 1966; Jenkins, 1967) with stannous chloride as a color reagent. These modifications are discussed in Fruh and Davis (1969). At least 90 per cent degradation of adenosine triphosphoric acid standards was obtained by both methods. The quantity of phosphorus in the bacterial cells was then computed by difference. Nitrogen determinations were made using a modified automated procedure (Fruh and Davis, 1969). The procedure of Von Hall, et al (1965) was followed to measure total organic carbon.

Microscopic examination was accomplished using a Bausch and Lomb microscope. Occasional checks of the bacterial population were made by the Gram staining technique. Albert's method for staining volutin (Frobisher, 1957) was utilized in some experiments as a check on cellular phosphorus determinations and alkaline phosphatase assays.

Photomicrographs of volutin stains were made using a Polaroid camera in conjunction with an American Optical Microstar Series 10

microscope equipped with an ortho-illuminator. Photomicrographs were taken at magnification of 1000 X using oil immersion. Volutin or polyphosphate inclusions are shown as dark masses in the cells.

Kinetics of the Alkaline Phosphatase Reaction

Effect of P-Nitrophenyl Phosphate (PNP) Concentration

There was a need to determine the PNP concentration to be used in the substrate so that this organic phosphorus source would not become limiting to the alkaline phosphatase. Equal volumes of bacterial suspension were filtered from a batch unit containing a low initial phosphorus concentration, and placed into six solutions containing PNP concentrations ranging from 0 to 600 mg/l. The p-nitrophenol produced was measured at various times following incubation at 35°C.

The slopes of the lines in Figure 1 give the rates of production of p-nitrophenol or the alkaline phosphatase activity. By determining the enzymatic activities from Figure 1 and plotting them against the initial PNP concentration as shown in Figure 2, the concentration at which PNP becomes limiting can be found. Above 25 mg/l of PNP, the enzymatic activity deviates from a linear dependence on PNP concentration.

Effect of Quantity of Alkaline Phosphatase

The limiting PNP concentration would no doubt be different at higher bacteria concentrations based on the logical premise that for the same environmental conditions 200 mg/l bacteria would have twice as much alkaline phosphatase as 100 mg/l bacteria. Experiments were conducted using different PNP concentrations and various bacterial concentrations. For the representative data presented in Figure 3, the experiment was conducted by filtering different volumes of bacterial suspension from the same batch unit, placing the bacteria in 100 mg/l PNP substrate, and measuring the quantity of p-nitrophenol formed at various incubation times.

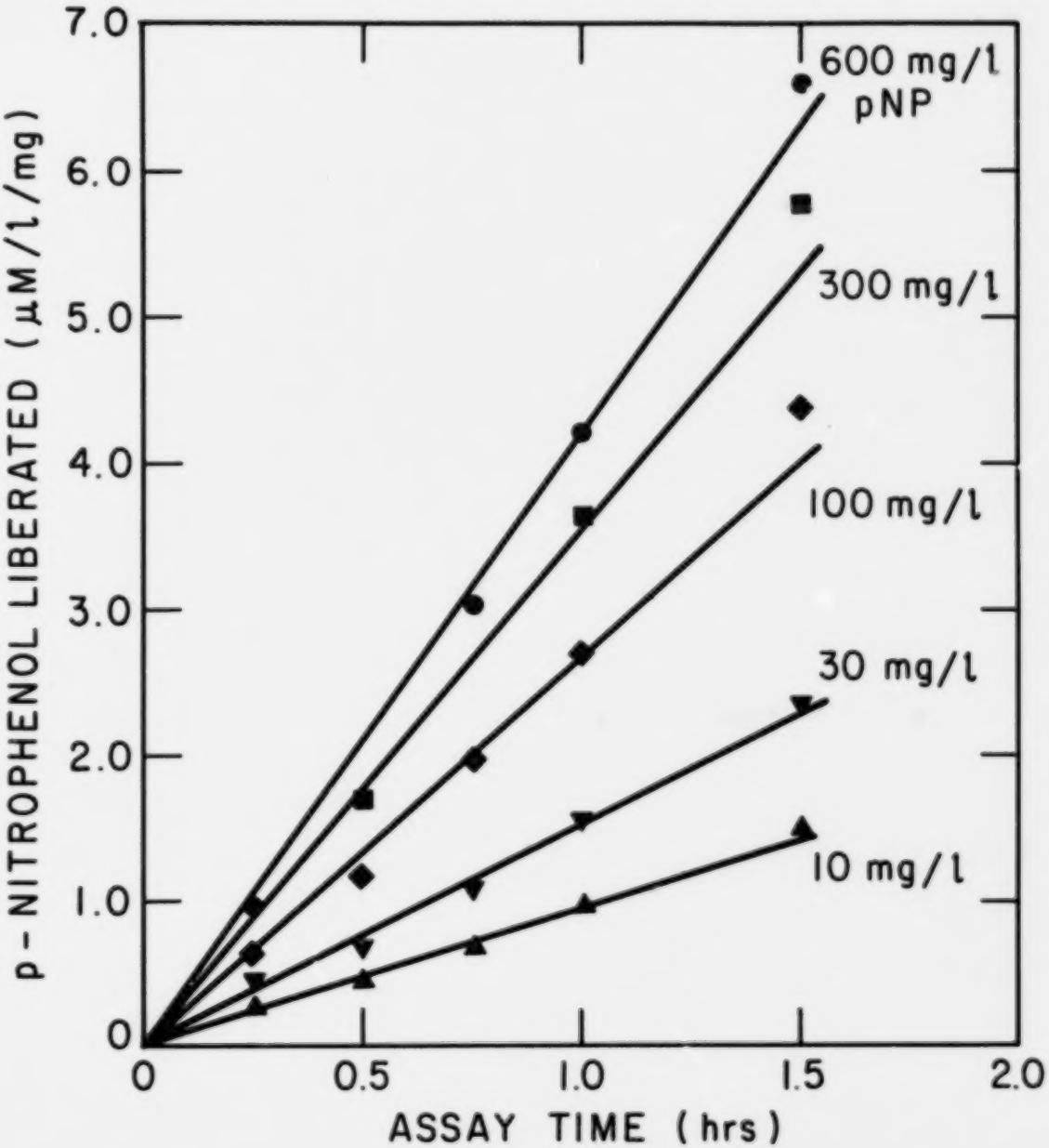


FIG. I. EFFECT OF INITIAL p-NITROPHENYL PHOSPHATE CONCENTRATION ON p-NITROPHENOL PRODUCED AT VARIOUS INCUBATION TIMES

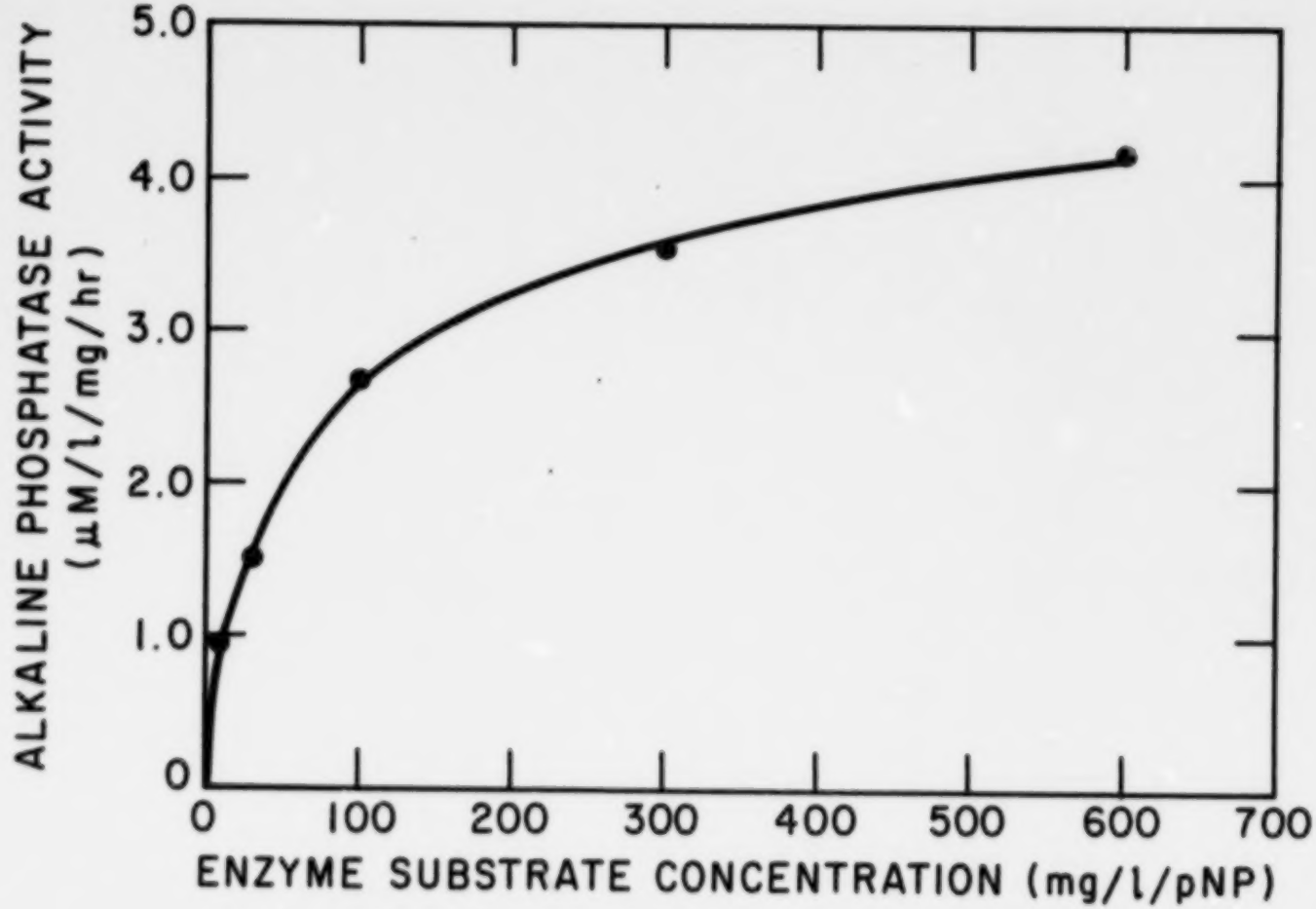


FIG. 2. EFFECT OF INITIAL p-NITROPHENYL PHOSPHATE CONCENTRATION ON ALKALINE PHOSPHATASE ACTIVITY

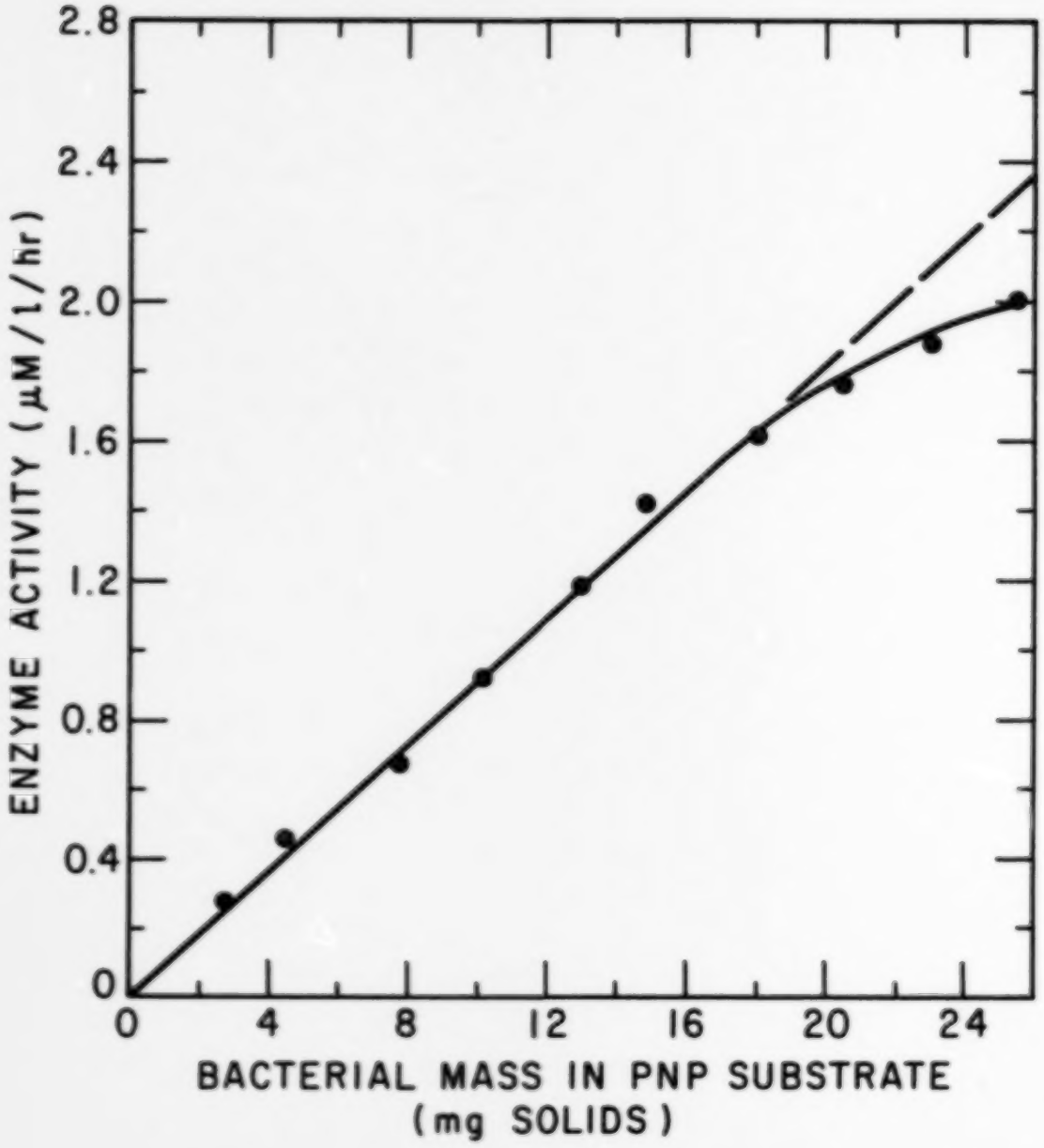


FIG. 3. EFFECT OF ENZYME CONCENTRATION ON ALKALINE PHOSPHATASE ACTIVITY

Although in Figure 2, 100 mg/l PNP was shown to be enzyme saturating for 7.1 mg. suspended solids, it is clearly shown in Figure 3 that above 20 mg. suspended solids, the alkaline phosphatase activity did not remain proportional to the enzyme quantity. In these particular experiments with the dilute bacterial cultures used, the problem was easily alleviated by increasing the initial PNP concentration to 500 mg/l.

Effect of Other Variables

Torriani (1960) indicated that for E. coli the optimum pH for the PNP substrate was between 8.8 and 9.8. In the experiments reported above, a pH of 9.0 was utilized. The objective of the following experiment was to determine the optimum pH of the PNP substrate for bacteria at various stages of growth. Bacteria for this experiment were obtained from two batch units; one with high phosphorus concentration, 30 mgP/l, and the other with low phosphorus concentration, 0.3 mgP/l. The bacteria in the batch units had been acclimatized to a pH of 8.3 to 8.5. The experimental results are tabulated in Table 1.

For all pH conditions, the alkaline phosphatase activity, as expected, was higher for the bacteria obtained from the batch unit with the lower initial phosphorus concentration. This difference was greater during the exponential growth portion of the bacterial life cycle. For the culture at the lower phosphorus concentration, the highest alkaline phosphatase activity was obtained in PNP solutions with a pH of 9.5 - 9.6. For the bacteria cultured at the higher phosphorus concentrations, the optimum pH of the PNP solution appeared to be between 8.5 and 9.5.

The objective of the second experiment was to determine if there was an interacting effect between the pH of the original batch unit and the pH of the PNP substrate. By varying the pH of both, any effect of acclimation was removed. The bacteria at the time of the analysis were in the exponential growth phase and the initial phosphorus concentration in the batch unit was 0.3 mg/l. The results of the experiment are presented in Table 2.

The most effective combination of pH values for the alkaline phosphatase test were a pH of 7.5 in the batch unit and a pH between 8.5 and 9.5 in the PNP substrate. The alkaline phosphatase activity decreased with increased pH in the original batch unit.

Repression of Alkaline Phosphatase Activity by Orthophosphate

The repression of alkaline phosphatase activity by orthophosphate was studied by placing different concentrations of orthophosphate into the PNP solution and measuring the quantity of p-nitrophenol formed with time. Bacteria used in this experiment were removed during their exponential phase of growth from a batch unit with an initial phosphorus concentration of only 0.1 mg/l .

The quantity of alkaline phosphatase activity showed marked decrease as the concentration of orthophosphate increased. (See Table 3.) The mixed culture from these experiments showed approximately the same repression as the E. coli of Torriani (1960) at an orthophosphate concentration about twenty times lower (See Figure 4.).

Table 1

INTERACTING EFFECT OF THREE VARIABLES ON ALKALINE PHOSPHATASE ACTIVITY

Growth Phase	pH of FNP Substrate	Enzyme Activity (μM/l/mg/hr) For Initial P in Batch Unit of 0.3 mgP/l	30.0 mgP/l
log	7.5	0.18	0.11
log	8.3	0.20	0.16
log	9.0	0.31	0.23
log	9.6	0.38	0.15
exponential	7.5	0.54	0.19
exponential	8.5	1.67	0.36
exponential	9.5	2.05	0.13

Table 2

INTERACTING EFFECT OF TWO DIFFERENT pH VARIABLES ON ALKALINE PHOSPHATASE ACTIVITY

pH of pNP Substrate	Enzyme Activity (μM/l/mg/hr) For pH of Batch Units of 7.5	8.5	9.5
7.5	1.54	0.67	0.09
8.5	2.86	1.49	0.26
9.5	3.95	1.77	0.09

Table 3

REPRESSION OF ALKALINE PHOSPHATASE ACTIVITY BY ORTHOPHOSPHATE

Orthophosphate Concentration (mgP/l)	Alkaline Phosphatase Activity (μM p-Nitrophenol/l/mg/hr)
0.0	4.85
0.5	4.60
5.0	2.20
50.0	0.50
500.0	0.20

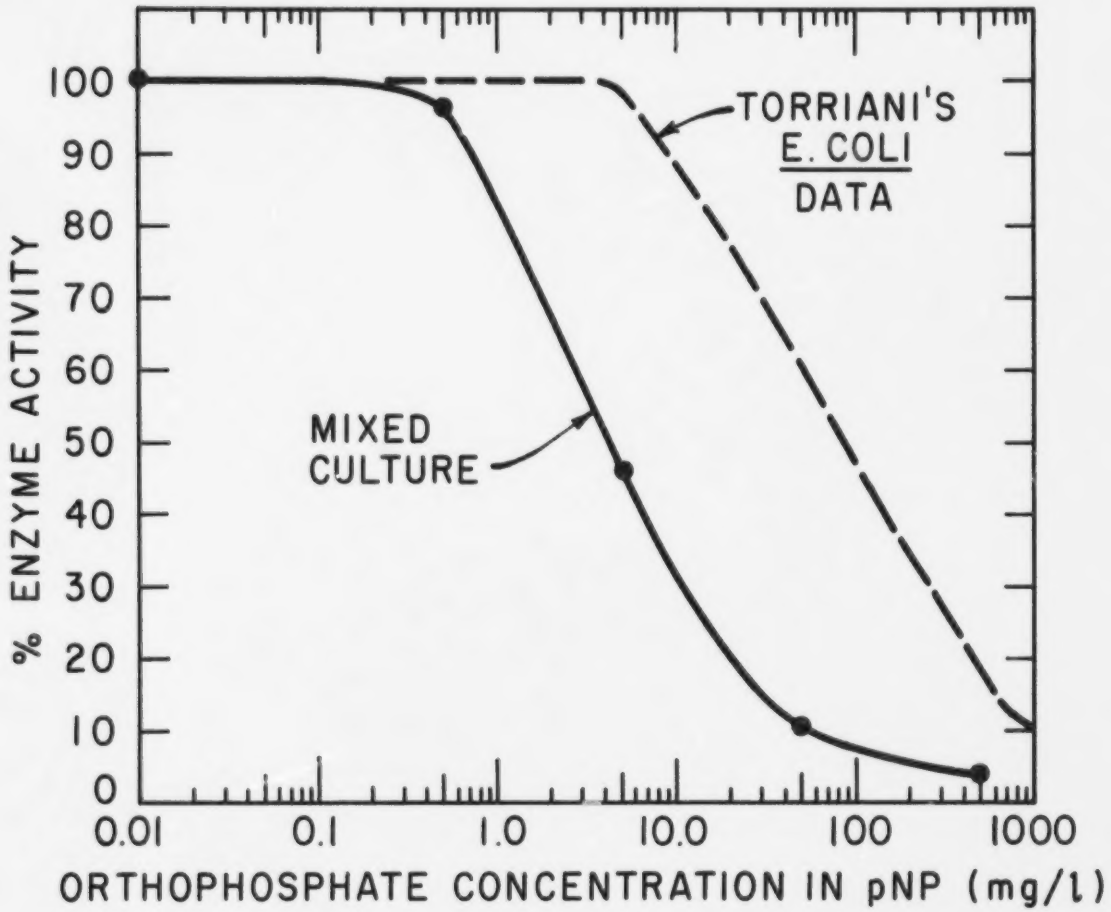


FIG. 4. EFFECT OF ORTHOPHOSPHATE IN THE pNP SUBSTRATE ON ALKALINE PHOSPHATASE ACTIVITY

Preliminary Observations

Relationship Between Initial Phosphorus Concentration and Alkaline Phosphatase Activity

Experiments previously discussed had shown that the alkaline phosphatase activity was higher for bacteria obtained from a batch unit containing an initial phosphorus concentration of 0.3 mgP/l than for bacteria cultured at 30.0 mgP/l. Further experiments were conducted in which the bacteria were removed at different stages of their life cycle from batch units containing five different initial phosphorus concentrations. Bacterial growth was measured by total suspended solids and optical density. Analyses for alkaline phosphatase activity, phosphorus , carbon and nitrogen were conducted. The growth curves are presented in Figure 5 and the experimental results are tabulated in Table 4.

As shown in Figure 5, growth was definitely limited for initial phosphorus concentrations of 0.0 and 1.0 mg/l. However, there was little difference in growth for the cultures having initial phosphorus concentrations from 3.0 to 20.0 mg/l. Evidently some other condition(s) of the environment other than phosphorus concentration was limiting the bacterial growth. Further experiments showed that addition of more nitrogen, sulfur and some trace elements increased the bacterial yield.

After 11 hours following incubation (the end of the "apparent lag phase"), there was three to four times more alkaline phosphatase activity in the bacteria cultured at 0.0 mgP/l than in the cells cultured at higher phosphorus concentrations. At this time only nitrogen (among the three nutrients measured) appeared to have been utilized to any degree.

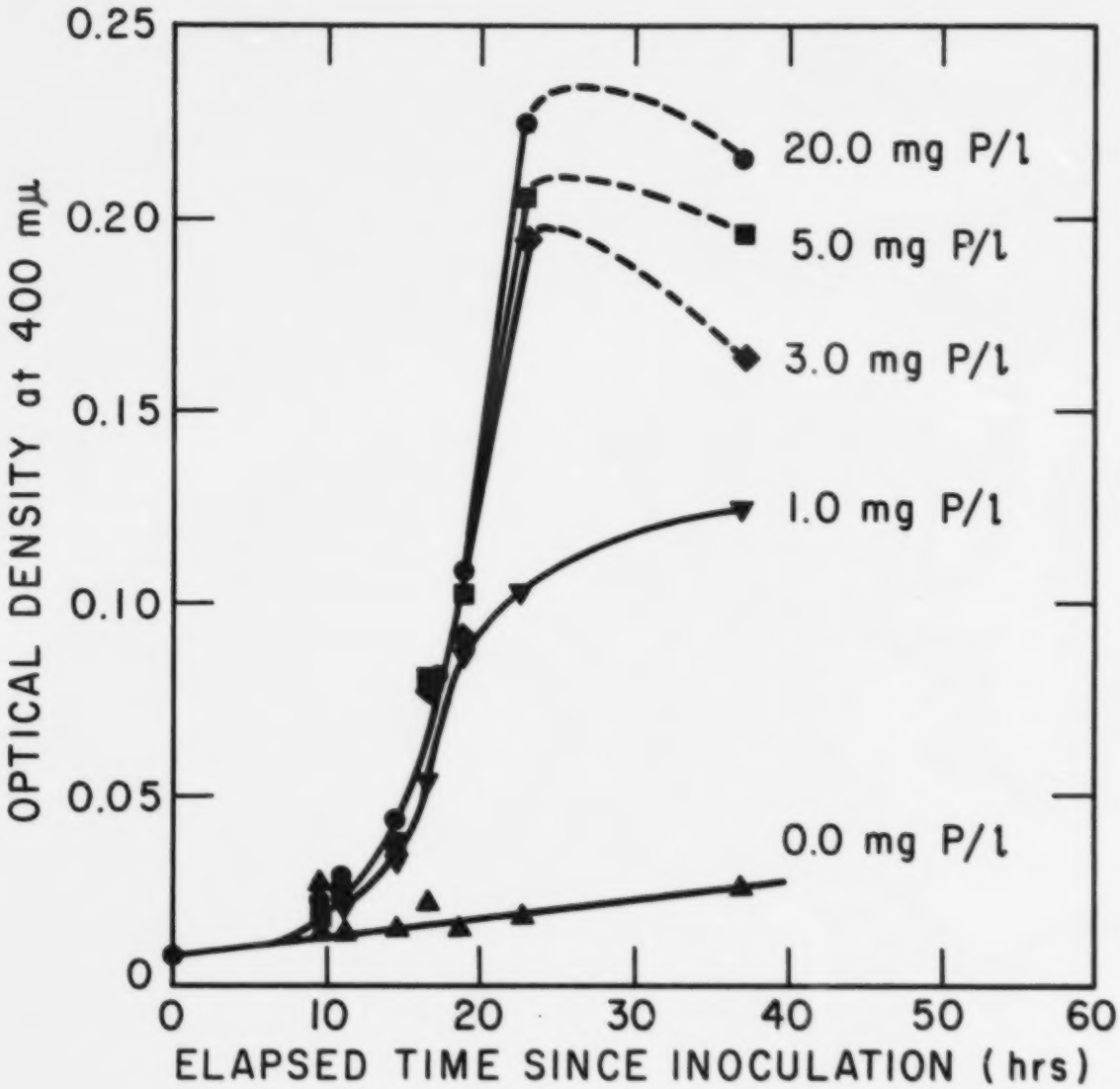


FIG. 5. GROWTH CURVES FOR BACTERIA CULTURED AT FIVE DIFFERENT INITIAL PHOSPHORUS CONCENTRATIONS

Table 4

PRELIMINARY OBSERVATIONS ON THE RELATIONSHIP BETWEEN "SURPLUS PHOSPHORUS UPTAKE" AND ALKALINE PHOSPHATASE ACTIVITY

Elapsed Time (Hours)	Batch Unit	Nutrients in Filtrate (mg/l) P	C	N	%P In Cells	Enzymatic Activity (μM/l/mg/hr)
0	1	0.0	323	20.3	--	--
	2	1.0	323	20.3	--	--
	3	3.0	323	20.3	--	--
	4	5.0	323	20.3	--	--
	5	20.0	323	20.3	--	--
11	1	0.0	323	20.6	--	1.03
	2	0.7	315	15.8	--	0.30
	3	2.8	323	16.3	--	0.35
	4	4.8	323	16.1	--	0.24
	5	20.0	322	--	--	0.24
22	1	0.0	323	13.3	--	2.83
	2	0.0	263	8.2	1.9	3.47
	3	0.3	238	4.5	3.4	0.30
	4	2.2	235	3.7	3.2	0.30
	5	17.4	228	7.7	2.8	0.30
37	1	0.0	323	12.1	--	0.94
	2	0.0	204	6.8	1.5	1.48
	3	0.1	175	6.6	2.4	0.35
	4	2.0	163	7.1	2.4	0.35
	5	17.3	155	5.5	2.2	0.35

At the end of 22 hours, the bacteria in all units had at least reached the end of their exponential growth phase. The bacteria cultured at an initial phosphorus concentration of 0.0 mg/l approximately tripled their alkaline phosphatase activity. Although there was no discernible utilization of carbon, approximately 7 mg/l nitrogen had been utilized. For the cells removed from the unit with an initial phosphorus concentration of 1.0 mg/l, the alkaline phosphatase activity had increased approximately 15 times as the phosphorus in the filtrate decreased to approximately 0 mgP/l. There was still phosphorus remaining in the other units with the higher initial phosphorus concentrations. The alkaline phosphatase activity of these units was similarly low. Because in the procedure the bacteria were filtered, washed, and then placed in the PNP solution which had no other source of phosphorus, only the phosphorus stored in the cells from units 3 through 5 could have caused the difference in the enzymatic activity obtained in comparison with units 1 and 2.

Approximately 37 hours after inoculation, all cultures were in the stationary or death phase. No apparent further utilization of nitrogen and phosphorus occurred in any unit, although carbon apparently was still being removed in units 2 through 5. The alkaline phosphatase activity in units 1 and 2 had decreased although it still remained higher than the activity found in units 3 through 5. Further analysis of these and other data indicated that this ambiguity was due to the inaccuracy of the suspended solids determination as a measure of living bacterial mass once stationary growth had been reached. The alkaline phosphatase activity of units 3 through 5 at 37 hours showed little change from that found after 11 hours growth. This further indicated storage of phosphorus and the limitation of growth shown in Figure 5 by some other nutrient such as nitrogen.

The carbon/nitrogen/phosphorus ratios in the cells of all units changed considerably during all phases of growth. A general correlation is evident between the per cent phosphorus in the suspended solids and the degree of alkaline phosphatase activity.

Effect of Orthophosphate Addition to Phosphorus-Starved Bacterial Cultures

In this experiment bacteria were cultured for 19 hours under "phosphorus starvation" conditions. At the end of 19 hours there was little growth, no soluble phosphorus left in solution, and the phosphorus content of the bacteria was 0.43 per cent. At this time, the above bacterial suspension was equally divided into five different batch units and phosphorus was added to bring the initial total phosphorus concentration to 0.40, 0.70, 1.20, 2.20 and 5.20 mgP/l, respectively. (Approximately 0.20 mgP/l of the above concentrations were present in the cellular material at the start of the experiment.) Analyses were conducted at subsequent times to determine alkaline phosphatase activity, suspended solids, optical density and total soluble phosphorus. Carbon and nitrogen concentrations were measured periodically to make sure that these compounds did not become growth limiting. The data obtained during this experiment are presented in Figures 6a - d. (On the time axis of all graphs, time 0 corresponds to the time at which different quantities of phosphorus were added to the phosphorus starved cells.)

The soluble phosphorus concentration in all five batch units decreased at a rapid rate as shown in Figure 6a. By 19 hours all the phosphorus had been utilized even in that unit which initially was increased by 5.0 mgP/l of orthophosphate. The growth curves of Figure 6b apparently indicate little growth (as measured by

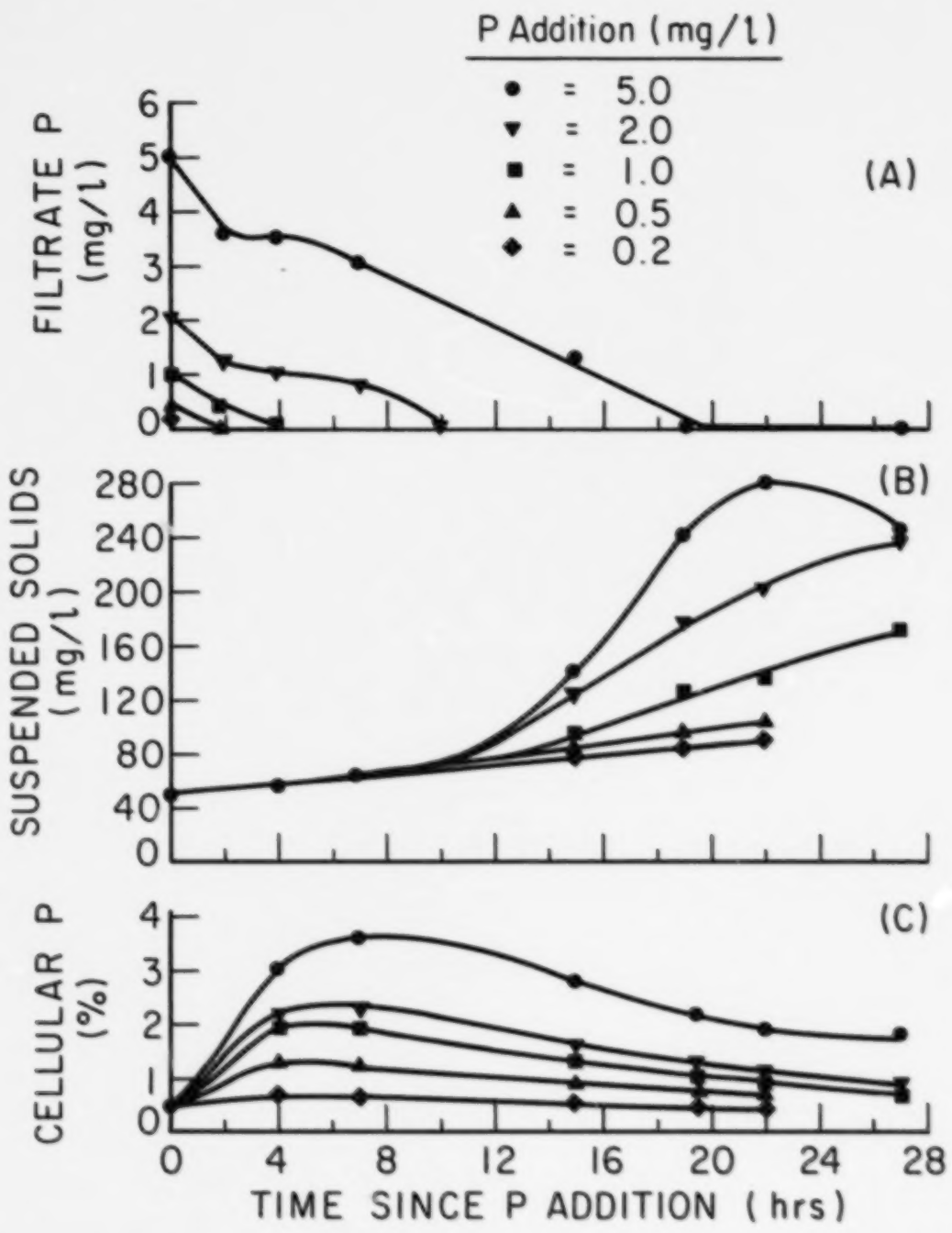


FIG. 6. PRELIMINARY OBSERVATIONS ON THE EFFECTS OF PO_4 ADDITION UPON A "P-STARVED" BACTERIAL CULTURE

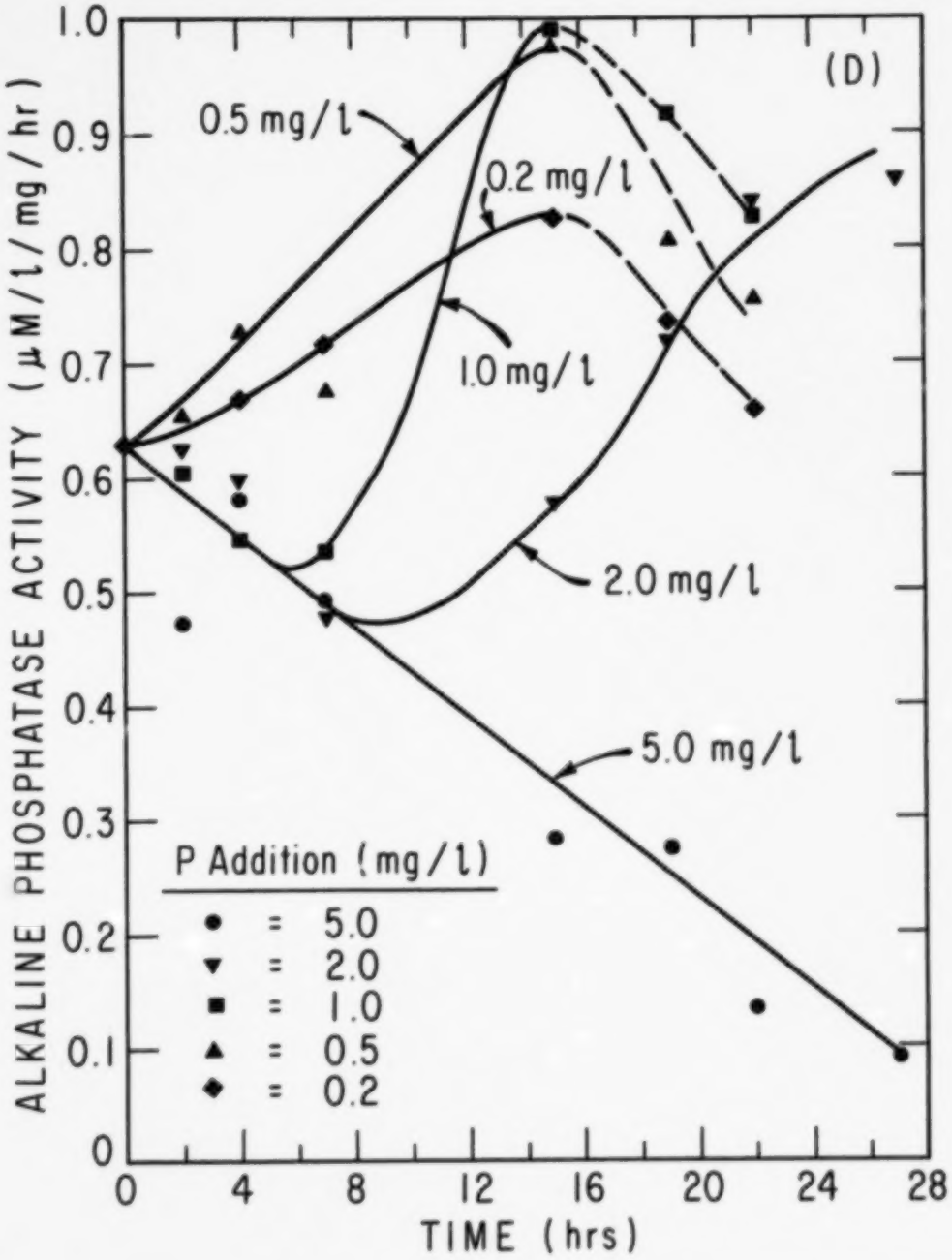


FIG. 6. PRELIMINARY OBSERVATIONS ON THE EFFECTS OF PO_4 ADDITION UPON A "P-STARVED" BACTERIAL CULTURE

suspended solids or optical density) in any of the units until eight to ten hours following the phosphorus addition. As shown in Figure 6c, the per cent phosphorus in the suspended solids apparently increased until about four to ten hours after the phosphorus addition, at which time it began to decrease. The point at which the per cent phosphorus in the suspended solids began to decrease coincided with the beginning of the rapid increase in suspended solids.

The unit to which 5.0 mgP/l had been added showed a rapid utilization of phosphorus and the per cent phosphorus in the suspended solids increased to 3.6 per cent. During this stage, the alkaline phosphatase activity decreased, but not as rapidly as the per cent phosphorus in the suspended solids increased. After ten hours the alkaline phosphate activity continued decreasing although the per cent phosphorus started decreasing. However, the phosphorus in the suspended solids never decreased below 1.75 per cent during the test.

The units with a phosphorus addition of 1.0 and 2.0 mg/l showed a decrease in alkaline phosphatase activity as the per cent phosphorus in the suspended solids initially increased. At 15 hours after the 1.0 mg/l phosphorus addition, the phosphorus in the suspended material had decreased to approximately 1.25 per cent and the alkaline phosphatase activity had increased. A similar relationsh o occurred a few hours later with the culture which had received and addition of 2.0 mgP/l.

The addition of 0.20 and 0.50 mgP/l was not above that quantity of phosphorus required by the cells for growth. The phosphorus in the suspended solids never was greater than 1.25 per cent and the alkaline phosphatase activity started increasing shortly after phosphorus addition.

However, the enzymatic activity for the 0.5 mgP/l spiking was higher than that obtained for the culture with the 0.2 mgP/l addition. Furthermore, following 15 hours after phosphorus spiking the enzymatic activity started decreasing for the cultures to which 0.2, 0.5 and 1.0 mg P/l had been added. Once again, experimental work indicated that suspended solids was not a valid measurement of bacterial activity in other than the exponential growth phase.

Experimental Results

Relationship Between Initial Phosphorus Concentration and Alkaline Phosphatase Activity

In this experiment, 10 milliliters inoculum were transferred from the acclimation unit (solids level approximately 2000 mg/l) to three batch units in which all conditions were the same except for initial phosphorus level. Phosphorus concentrations of 0.3, 3.0, and 30.0 mgP/l were employed. After inoculation, bacterial growth was allowed to develop in all units as shown in Figure 7. Volatile suspended solids measurement was used as an indication of bacterial growth in order to avoid previous difficulties in the use of total suspended solids content. At various times in the life cycles of the dilute cultures, alkaline phosphatase activity, cellular phosphorus content, total soluble phosphorus remaining in culture filtrate, TOC, ammonia and nitrate-nitrite, and volutin measurements were made. Experimental results are presented in Table 5.

As illustrated in Figure 7, volatile suspended solids levels were low for approximately 30 hours. At this time, growth in all three units entered exponential phase. At 39 hours, alkaline phosphatase was measurable in all three units. As indicated in Table 5, enzyme activity was highest for the unit with lowest available phosphorus supply. The cellular phosphorus content was likewise lowest in cells from the unit with high enzyme activity. This emphasizes the ability of extracellular and internally stored phosphorus to regulate alkaline phosphatase formation.

At 54 hours after inoculation, the unit with 0.3 mgP/l had entered stationary growth while the other two units were in declining growth phase. The cellular phosphorus content had decreased for all

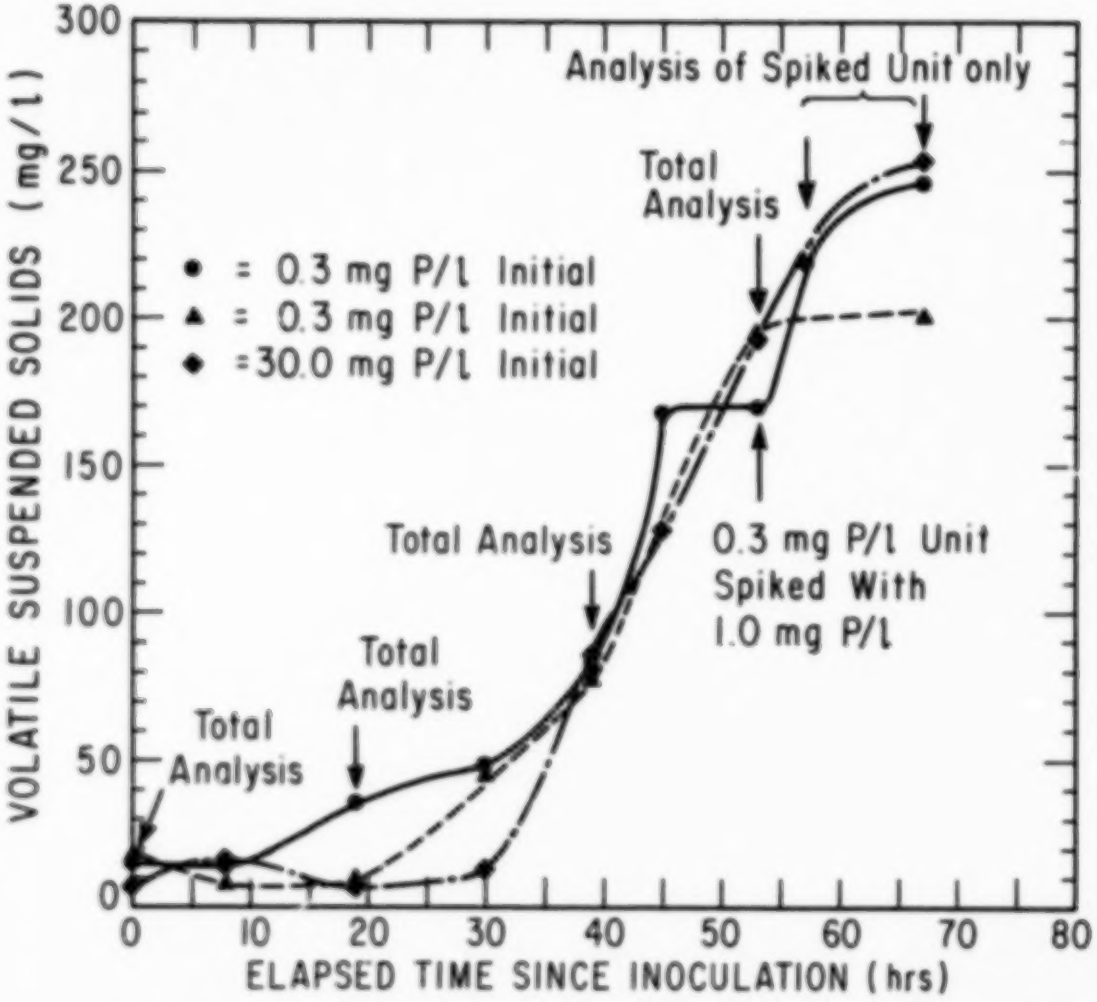


FIG. 7. BACTERIAL GROWTH OF CULTURES WITH DIFFERENT INITIAL PHOSPHORUS CONCENTRATIONS

Table 5

EXPERIMENTAL RESULTS ON THE RELATIONSHIP BETWEEN THE INITIAL PHOSPHORUS CONCENTRATION AND ALKALINE PHOSPHATASE ACTIVITY

Elapsed Time (hrs.)	Sample	VSS (mg/l)	Enzyme Activity (M/l/mg/hr)	C (mg/l)	NH_3 (mg/l)	$NO_3 - NO_2$ (mg/l)	P (mg/l)	Cellular P (%)	Volutin* No.	Volutin* Size
0	0.3 mgP/l			356	25	.13	0.240		+++	+++
	3.0			338	25	.13	2.45		+++	+++
	30.0			348	25	.13	30.0		+++	+++
19	0.3			332	25	.14	0.030		+++	++
	3.0			348	25	.14	2.05		+++	++
	30.0			348	25	.14	27.0		+++	+++
30	0.3	48.3								
	3.0	45.4								
	30.0	13.0								
39	0.3	81.0	8.27	290		.13	0.015	.352	++	+
	3.0	77.5	5.03	92		.10	0.200	3.85	++	+
	30.0	86.0	1.10	200		.08	17.8	14.2	++	++
45	0.3	168.0								
	3.0	228.0								
		127.0								

Table 5 (cont'd)

EXPERIMENTAL RESULTS ON THE RELATIONSHIP BETWEEN THE INITIAL PHOSPHORUS CONCENTRATION AND ALKALINE PHOSPHATASE ACTIVITY

PO_4 Addition to Phosphorus - Starved Bacterial Cultures

Elapsed Time (hrs.)	Sample	VSS (mg/l)	Enzyme Activity (M/l/mg/hr)	C (mg/l)	NH_3 (mg/l)	$NO_3 - NO_2$ (mg/l)	P (mg/l)	Cellular P (%)	Volutin* No.	Volutin* Size
53	0.3	169.0	8.60	252		.17	0.038	.155	+	+
	3.0	195.0	7.14	88		.01	0.725	1.17	+	+
	30.0	193.0	2.07	138		.10	19.0	5.70		
53	1.0 mgP/l Added to Unit with 0.3 mgP/l Initial.									
57	0.3 & 1.0	219.0	3.03	228		.14	0.088	.556	+++	++
67	0.3 & 1.0	246.0	8.09	190		.10	0.010	.524		

* Coding for Volutin Content of Bacterial Cells:

No. of Granules	Granule Size
+++ = Large Number	+++ = Large Size
++ = Medium Number	++ = Medium Size
+ = Small Number	+ = Small Size
0 = None	0 = None

three units since the 30 hour measurement. As expected, this decrease in stored P content was accompanied by an increase in enzyme activity for all three cultures. The standard pattern of highest alkaline phosphatase activity for the culture with lowest available phosphorus supply and cellular content still prevailed.

Following measurements at 53 hours, a 1.0 mgP/l addition was made to the unit with initial phosphorus level of 0.3 mgP/l. Analysis at 57 hours demonstrated a large buildup of volatile suspended solids due to the phosphorus rejuvenation of the P-starved bacteria. Cellular level of the element had also increased due to the increased availability of phosphorus. Alkaline phosphatase activity had decreased considerably as expected. At 67 hours, bacteria in the unit were again becoming phosphorus limited, growth was declining, phosphorus availability had decreased, and hence, alkaline phosphatase activity was considerable. Cellular phosphorus content had become less since the 57 hour determination, but the slight decrease accompanied by the large enzyme increase indicates a lag period between cellular phosphorus change and enzyme response. The relationship between enzymatic activity and per cent phosphorus in the cell as well as the lag effect of P addition on enzymatic activity are demonstrated in Figure 8.

The volutin staining procedure for stored phosphorus indicated a pattern of decrease in both number and size of polyphosphate granules as growth progressed. The addition of phosphorus to the limited culture resulted in an increase in granule size and number. (See Figure 9.) The volutin observations served, then, as a rough check on the alkaline phosphatase and cellular phosphorus measurements.

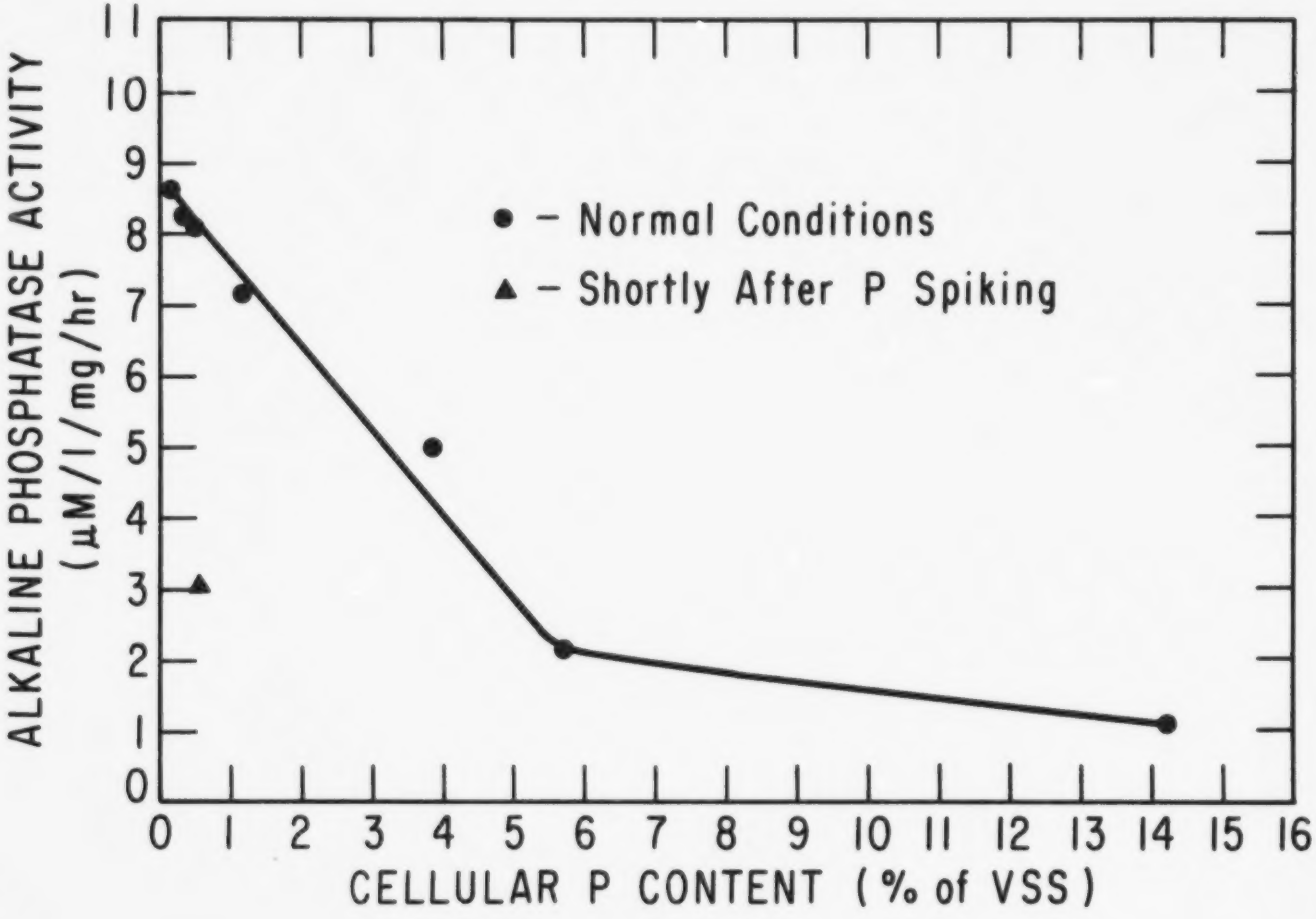
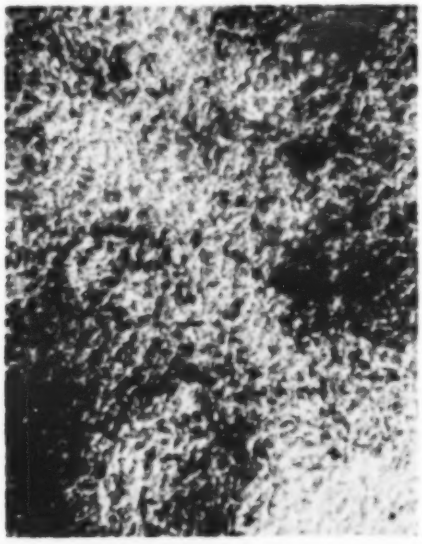
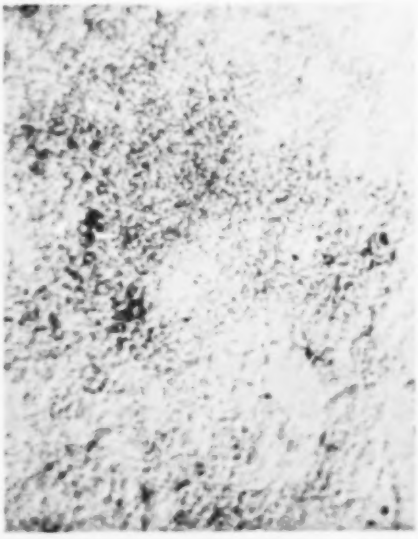


FIG. 8. RELATIONSHIP OF ENZYME ACTIVITY AND CELLULAR PHOSPHORUS CONTENT IN DILUTE BACTERIAL CULTURES

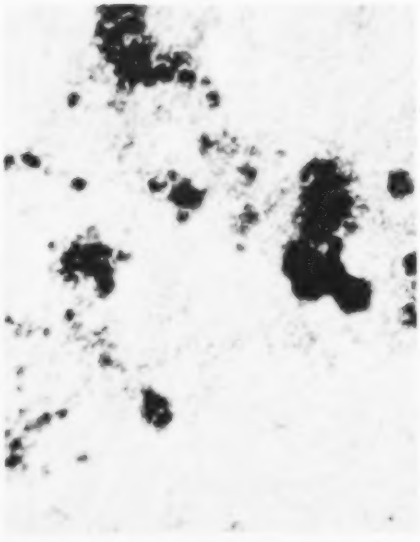
19 hours
0.3 mg P/l initial



39 hours
0.3 mg P/l initial



53 hours
0.3 mg P/l initial



57 hours (4 hrs after P addition)
0.3 mg P/l initial + 1.0 mg P/l addition

FIG. 9. PHOTOMICROGRAPHS OF VOLUTIN STAINS FOR POLYPHOSPHATE IN BACTERIAL CELLS

Effect of Orthophosphate Addition to Phosphorus-Starved Bacterial Cultures

A second experiment was then conducted under the same conditions as employed in the previous experiment except that the three batch units were all provided initially with a phosphorus level of 0.3 mgP/l. The purpose of this study was to determine the effect of a wider range of orthophosphate additions on the alkaline phosphatase activity of P-starved bacteria. After inoculation with a small quantity of microorganisms from the acclimation unit, volatile suspended solids development was monitored to indicate growth and analyses were accomplished as shown in Figure 10. The alkaline phosphatase activity data are presented in Figure 11.

Alkaline phosphatase activity at 40 hours after inoculation was relatively high since volatile suspended solids indicated the cultures were in late exponential or early declining growth phase and were apparently becoming P-limited. The enzyme activity in the three cultures under the same conditions compared favorably.

All three cultures were in stationary phase at 55 hours, and alkaline phosphatase had risen considerably due to phosphorus deficiency. Phosphorus additions of 0.3, 1.0, and 5.0 mgP/l were made to the three units at 57 hours elapsed time. The phosphorus additions caused immediate increase in the volatile solids in all three units. Enzyme activity was repressed in all three cultures at a rapid rate following the phosphorus spiking. Eighteen hours after phosphorus addition, the microflora spiked with 0.3 mgP/l had again become phosphorus deficient as indicated by VSS measurement, and, hence, alkaline phosphatase activity was beginning to rise. The activity continued to increase and returned to its highest level by 43 hours after spiking (100 hours total elapsed time).

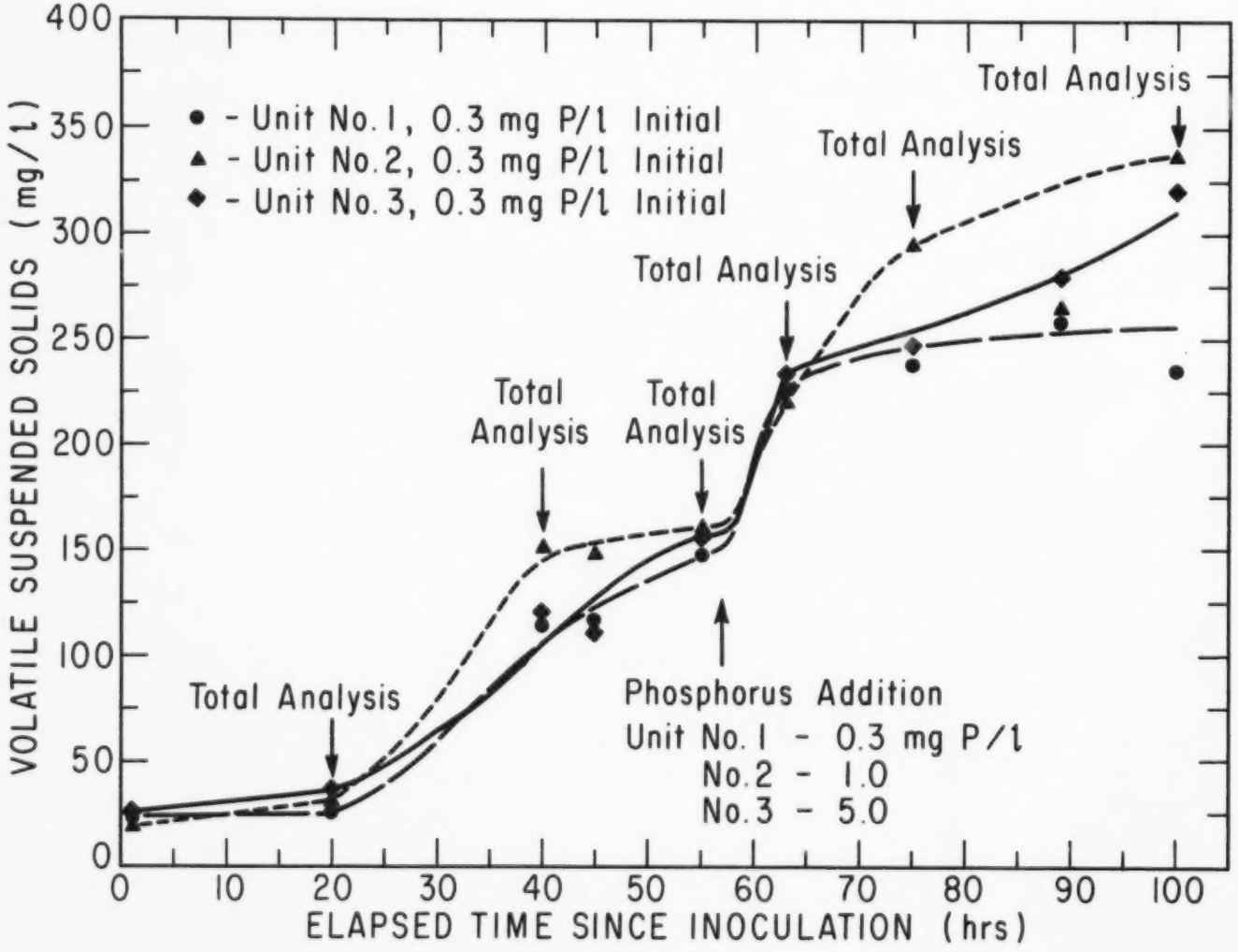


FIG. 10. EFFECT OF PO_4 ADDITION UPON GROWTH OF "P-STARVED" BACTERIAL CULTURES

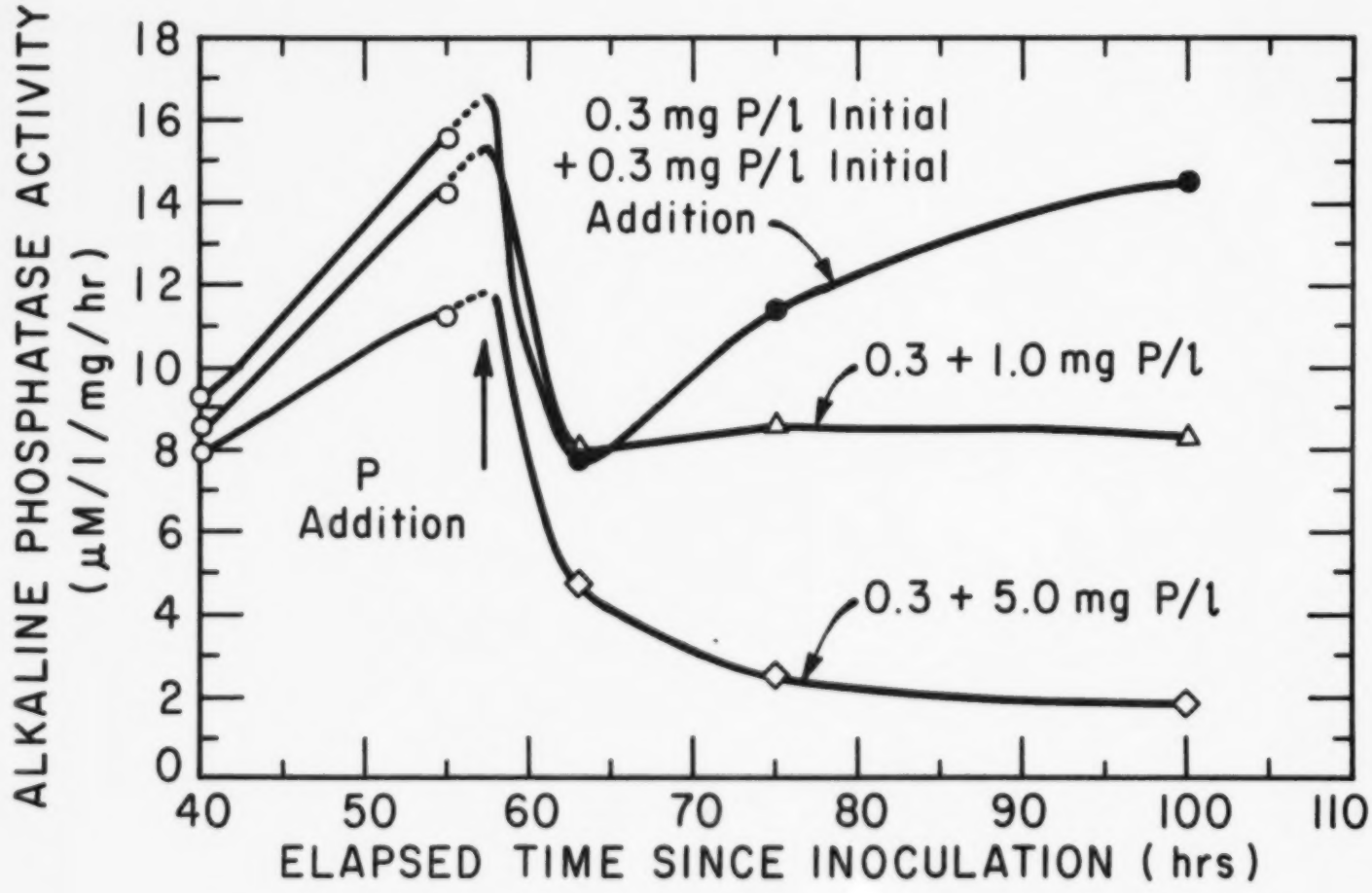
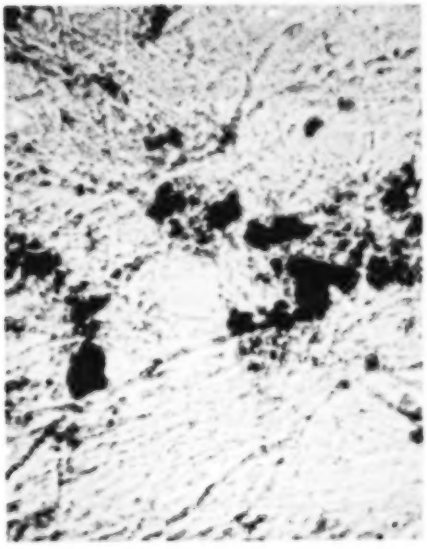


FIG. II. EFFECT OF VARIABLE P ADDITIONS ON ALKALINE PHOSPHATASE ACTIVITY

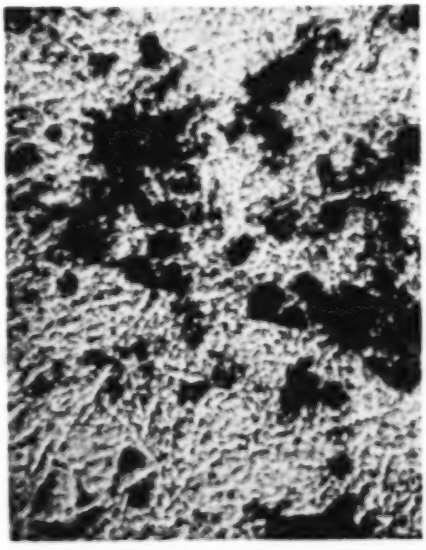
The activity in the unit to which 1.0 mgP/l was added demonstrated a rapid decrease after the addition and the remained constant at the decreased activity. Addition of 5.0 mgP/l to the third unit resulted in continued decline in alkaline phosphatase activity from time of phosphorus spiking to time of final measurement at 100 hours total elapsed time.

Volutin observations in this experiment (see Figure 12) again indicated decrease of number and size of phosphorus inclusions as growth progressed and then return to larger size and greater number after phosphorus additions. Gram staining demonstrated the predominant organisms present to be Gram negative rods. The organisms appeared to become increasingly filamentous as time in the batch unit progressed.

20 hours
0.3 mg P/l initial



55 hours
0.3 mg P/l initial



75 hours (18 hrs after P addition)
0.3 mg P/l + 1.0 mg P/l addition

100 hours (43 hrs after P addition)
0.3 mg P/l initial + 1.0 mg P/l addition

FIG. 12. PHOTOMICROGRAPHS OF VOLUTIN STAINS FOR POLYPHOSPHATE IN BACTERIAL CELLS

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Discussion and Conclusion

An enzymatic procedure was developed in this study to determine during phosphorus removal what was occurring in the cells of dilute bacterial cultures which were obtained from an activated sludge process. The procedure which utilized a p-nitro phenyl phosphate substrate incubated at 35°C was quick and reliable. The kinetics of the alkaline phosphatase reactions fulfilled the requirements of typical enzymatic assays. The concentration of p-nitrophenyl phosphate limiting to the enzyme was found to be a function of the quantity of enzyme present. The pH of the p-nitrophenyl phosphate most useful for all phosphate concentrations used to culture the bacteria appeared to be at pH 9.0. Bacteria cultured in the typical pH range found in biological treatment units had greater alkaline phosphatase activity than those bacteria acclimated to higher pH values. The presence of orthophosphate external to the bacterial cells was demonstrated to repress formation of alkaline phosphatase. This repression of the enzyme by orthophosphate was found to be greater for the mixed bacterial cultures obtained from activated sludge units than for pure cultures of bacteria (Torriani, 1960).

The alkaline phosphatase enzyme procedure was found to be an indicator of "surplus" phosphorus uptake by the bacteria. This indication was shown by the fact that under the procedures of this enzymatic test only "surplus" phosphorus storage could repress the alkaline phosphatase activity. Thus, a high enzymatic activity was indicative of little or no phosphorus storage and low enzymatic activity indicated phosphorus storage beyond metabolic needs of the bacteria. The relationship of alkaline phosphatase activity and cellular phosphorus content obtained from the data in Table 5 for the dilute

bacterial cultures is shown in Figure 8. The storage of phosphorus within the bacterial cells was also demonstrated by a Microscope staining procedure for detection of volutin or polyphosphate granules (Frobisher, 1957; Stanier, et al, 1963). Selected photomicrographs of dilute bacterial stains for volutin are shown in Figure 9 and 12.

Certain problems, however, did occur in the preliminary experiments. The reason for these discrepancies was theorized to be the measurement of the quantity of living bacterial mass. Throughout the preliminary experiments, the suspended solids content in the bacterial units was used for this measurement mainly so comparison could be made with the results of previous investigators (Fitzgerald and Nelson, 1966; Fruh and Pessoney, 1969) who used suspended solids as a measure of living algal mass. The alkaline phosphatase activity of algae and bacteria are, in fact, similar. However, it is apparent from this study that suspended solids are not a reliable measurement of living bacterial mass, particularly during the lag and stationary growth phases. Consequently, the use of volatile suspended solids analysis as a growth indicator was incorporated into the second and third "phosphate addition" experiments. Expression of alkaline phosphatase activity results on a volatile suspended solids basis rectified earlier difficulties.

Continuing Research

Based on the findings of this research, it is expected that the alkaline phosphatase bioassay can be used to help differentiate the various mechanisms of phosphorus removal occurring in some activated sludge units. In such a procedure, the mixed liquor suspended solids would be removed from the aeration unit, filtered and placed in the appropriate concentration of PNP (utilizing the procedure outlined in this study). If a low alkaline phosphatase activity and a high per cent phosphorus in the sludge was found, the mechanism of phosphorus removal would probably be "surplus" phosphorus uptake. This could be checked by a staining procedure for volutin. If a high enzymatic activity and a high per cent phosphorus in the sludge was found, the mechanism of phosphorus removal would probably be chemical precipitation or physical sorption of the phosphorus on the surface of the sludge.

To test this hypothesis, research is being or will be conducted using:

1. Batch laboratory tests with concentrations of activated sludge normally found in wastewater treatment plants;
2. "Shock" additions and dilutions of phosphorus to continuous flow laboratory units; and
3. Field testing at the San Antonio wastewater treatment plants.

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